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Phosphoprotein progesterone receptor (PR) is a key mediator of sex hormone progesterone, which regulates the development and differentiation of many organs including mammary gland. Aberrant activity of PR may be involved in breast cancer development. We have been interested in the effect of phosphorylation on PR activity and antagonist activity of RU 486 to agonist activity switch. We sought to first identify phosphorylation sites in human PR in T47D breast cancer cells so that the significance of phosphorylation of individual site can be studied using site-directed mutagenesis approach. Nine phosphorylation sites were previously identified. Our current studies revealed a new site and one more site remains to be identified. The effect of several signal transduction pathways on the RU 486 antagonist activity to agonist activity switch has been tested. RU 486 became a stronger antagonist in the presence of epidermal growth factor and ras oncogene, suggesting that multiple pathways can contribute to the agonist activity of RU 486. I have also compared the activity of wild type hPR with mutants Ala400 and Glu400. The transcriptional activity of Ala400 was much lower than that of the wild type, while Glu400's activity was comparable to wild type. The mutagenesis studies suggest that phosphorylation is important for the activity of hPR.

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Gillian Harvey 7/28/97

PI Signature Date

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I. Introduction

Breast cancer is the second leading cause of cancer death in women. Despite extensive research efforts, the causes of breast cancer remain largely unknown; the current means of prevention and therapies are not entirely effective. At present, the major endocrine therapy for breast cancer utilizes the antiestrogen tamoxifen, and antiprogestin RU486. Since the effectiveness of endocrine treatment requires functional receptors, it is crucial to determine what regulates the activity of the receptors.

Human progesterone receptor (hPR) is a specific mediator of the activity of progesterone, an important regulator of growth and development of the human mammary gland. hPR belongs to a superfamily of ligand-activated nuclear transcription factors and consists of two forms, hPR-A (Mr = 94,000) and hPR-B (Mr = 120,000) resulting from differential transcription of a single gene (1,2). hPR-A differs from hPR-B in that hPR-A lacks the first 164 amino acids (a.a.) of hPR-B. Like other members of the family, hPR contains a highly conserved DNA binding domain, a ligand binding domain, and a variable amino-terminal region, which contains a transactivation domain (AF1). There is also a hormone dependent activation domain (AF2) in the carboxyl terminus. Sartorius et al. (3) recently showed that the first 164 a.a. of hPR-B is an independent transactivation domain which they named AF3. Activation of hPR by progestin in target tissues is mediated via a series of yet to be learned events that include at least dimerization and phosphorylation of the receptor, receptor interacting with other factors, and receptor binding to PR responsive elements on target genes. The effect of progestin can be blocked by antiprogestins, which leads to the potential use of antiprogestin for treating breast cancer. However, like an estrogen antagonist tamoxifen, which is also a partial agonist for estrogen receptor, agonist activities of antiprogestin have been reported (4,5). In cultured breast cancer cell lines, RU 486 behaves as an agonist in the presence of a protein kinase A activator, 8-Br cAMP (6,7).

The goals of this research are to understand the role of phosphorylation in modulating hPR activity and the mechanisms underlying the agonist effect of RU 486 in the presence of 8-Br cAMP. To accomplish these goals, we decided to first identify the phosphorylation sites in hPR and to investigate each individual site concerning its biological function and relation to this problem.

hPR is a phosphoprotein and is highly phosphorylated in T47D breast cancer cells through multiple steps upon hormonal stimulation (8,9). The rapid phosphorylation takes place in a few minutes after the addition of hormone. Prolonged treatment up to 60 min does not change the overall ^{32}P incorporation significantly, but decreases the mobility of hPR (upshift) on SDS gel electrophoresis (SDS-PAGE), indicating that phosphorylation can cause significant conformational changes within hPR. In the previous report, I demonstrated the identification of multiple phosphorylation sites; three are hormone inducible sites (Ser102, Ser294 and Ser345) and their phosphorylation correlates with the timing of the change in mobility on gel electrophoresis in response to hormone treatment (10). The other sites, Ser20, Ser81, Ser162, Ser190, Ser400, Ser558 are basally

phosphorylated and their phosphorylation exhibits a rapid increase in response to hormone (10,11,1996 report).

All identified sites except Ser81 contain a Ser-Pro consensus sequence, a motif for proline-dependent kinases such as MAP kinases and Cdks. Using purified baculovirus expressed hPR-B as a substrate, Ser190 and S400, as well as another basal site Ser162 were specifically phosphorylated to a high specific activity *in vitro* by Cdk2 while Ser81 is specifically phosphorylated by casein kinase II (11). Thus *in vitro* phosphorylation studies suggest that hPR is phosphorylated by multiple kinases. The physiological significance of these findings needs to be assessed by future *in vivo* studies.

Phosphorylation is a key process that modulates functions of many proteins. Several lines of evidence have shown that phosphorylation of steroid receptors affects the binding of receptor to hormone onto DNA (12,13,14), and receptor nucleocytoplasmic shuttling (15). However, *in vivo* functional analysis remains incomplete and only a few studies have been documented. Until now, our lab has identified four phosphoserines in chicken PR (cPR) (16,17). Mutation of the hinge Ser^{530} phosphorylation site (between the DNA-and ligand-binding domain) to Ala decreases hormone sensitivity, implying that phosphorylation of this site is important for transcriptional activity at physiological levels of hormone (18). In addition, mutation of Ser^{211} to Ala reduced transcriptional activity of cPR dramatically (19). Western analysis of the Ser^{211} mutant showed that the level of upshift was reduced, inferring that phosphorylation of Ser^{211} affects the conformation of cPR. Significant reduction of transcriptional activity was also reported for an estrogen receptor mutant (20). In contrast, initial studies on mutant glucocorticoid receptor (GR) suggested that although phosphorylation affected the stability of GR, effect on transcriptional activity was insignificant (21). However, more recent studies on GR mutants have shown that certain phosphorylation sites when mutated do affect several functions of receptor (22). Previously, I have shown that mutation of Ser81 to Ala caused significant decrease of hPR activity when compared with wild type, confirming that phosphorylation is important for hPR function. It is one of our long term goals to characterize the functional role of the remaining phosphorylation sites in hPR.

Several possible mechanisms underlying the RU 486 antagonist to agonist activity switch were proposed. To address the question of whether RU 486 plus 8-Br cAMP can alter or induce new sites in hPR, we have also performed phosphotryptic mapping experiments. Our studies showed that there is no altered phosphorylation or any new site induced by RU 486 and 8-Br cAMP (1995 report), suggesting that the agonist activity of RU 486 is not due to the phosphorylation of receptor itself. Therefore, we speculated that the agonist activity is mediated through hPR-associating proteins, whose activity is perhaps modulated by cAMP-mediated phosphorylation. The recent demonstration of co-regulators of steroid receptors by our group and others allows me to test whether these co-regulators play any part in the switch. In my 1996 report, I showed that SRC-1 potentiates the PKA mediated activation of hPR in the presence of R5020. It is of profound interest to test whether or not it also affects the switch. Regretfully, this is beyond the scope of my proposed project and last year's reviewer also pointed that out.

Therefore I decided not to pursue co-regulators and their involvement in the switch during this reporting period. Interestingly, two recent reports demonstrated that co-repressor N-CoR may play a role in suppressing the agonist activity of RU 486 and 8-Br cAMP is able to release the repression (23,24), supporting that our speculation about the involvement of co-regulators in the switch is correct.

In this report, I conclude the identification of phosphorylation sites. I demonstrate that growth factor and oncogene can also participate in the agonist activity of RU486. Finally, I compare the transcriptional activity of wild type with phosphorylation site mutants Ala400 and Glu400.

II. Body

A. Materials and Methods

Materials

Minimum essential medium (MEM) was purchased from Irvine (Santa Ana, CA). Phosphate-free MEM was obtained from GIBCO BRL (Grand Island, NY). AB-52 mouse monoclonal antibody that recognizes both PR-A and PR-B is provided by Dr Dean Edwards. R5020 and carrier-free [^{32}P]H_3PO_4 were purchased from Dupont/New England Nuclear Products (Boston, MA). Protein-A Sepharose was purchased from Pharmacia LKB Biotechnology, Inc. (Piscataway, NJ). Tosylphenylalanyl chloromethy ketone-treated trypsin was purchased from Worthington Biochemical Corp. (Freehold, NJ). Sequencing grade endoproteinases Asp-N and Glu-C, and lipofectamine transfection reagent were purchased from Boehringer Mannheim (Indianapolis, IN). Phenylisothiocyanate and sequencing grade trifluoroacetic acid (TFA) and HPLC reagents were purchased from J. T. Baker Chemical Corp. (Phillipsburg, NJ). Triethylamine and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) were purchased from Sigma (St. Louis, MO). Sequelon-AA membranes and Mylar sheets were obtained from Millipore Corp. (Milford, MA). Transformer™ site-directed mutagenesis kit was obtained from Clontech (Palo Alto, CA). Sequenase version 2.0 DNA sequencing kit was obtained from USB (Cleveland, Ohio).

Cell Culture, PR labeling, and Receptor Preparations T47D human breast cancer cells were maintained and grown in 75-cm^2 T-flasks with frequent changes of media as previously described. Cells were incubated for 24 h in MEM containing 5% fetal calf serum that has been stripped of steroid hormones by dextran-coated charcoal treatment. Steady state labeling with [^{32}P]orthophosphate was carried out in phosphate-free serum-free medium for 1 h at 37^{0}C and then incubated in phosphate-free MEM containing [^{32}P]orthophosphate (0.83 mci/ml) for 6 h at 37^{0}C. Cells were treated with 40 nM R5020 for 2 h before harvest.

Cells were harvested in 1 mM EDTA in Earle's balanced salt solution and homogenized at 40^{0}C in a Teflon-glass Potter-Elvenhjem homogenizer (Fisher, Pittsburgh, PA) in KPFM buffer [50 mM potassium phosphate (pH 7.4), 50 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, and 12 mM monothioglycerol] containing 0.5 M NaCl and a mixture of proteinase inhibitors as previously described. The whole cell extract was obtained by centrifuging the homogenates at 100,000 x g for 30 min, and dialyzed in KPFM to remove the salt before the immunoprecipitation step.

Immunoprecipitation and Gel Purification of PR. Monoclonal antibody AB-52 bound Protein-A Sepharose was prepared as previously described. Dialyzed whole-cell extracts containing PR were incubated with Protein-A Sepharose on an end-over-end rotator for 4 hours at 4^0 C. Protein-A Sepharose was washed three times with KPFM buffer containing 0.3 M NaCl to remove nonspecific protein. Bound receptors were then eluted with 2% SDS sample buffer and electrophoresed on a 7.0% discontinuous SDS

polyacrylamide gel. ^{32}P-labeled receptors were located by autoradiography of the gels, and PR-A and PR-B were retrieved by excising the corresponding gel pieces.

HPLC Analysis of Tryptic Peptides of PR. The gel slices containing PR were washed with 50% methanol for 1 hour followed by H_2O for 30 min and 50 mM ammonium bicarbonate for 5 min in a 1.5 -ml microfuge tube. 20 ug trypsin was added to the tube. After incubating the tube for 4 hours at 37°C, another 20 ul trypsin was added, and this was repeated three more times. The digested peptides were dried in a Speedvac (Savant Instruments, Hicksville, NY), dissolved in 150 ul 50% formic acid, loaded on a Vydac (Hesperia, CA) C18 reverse phase column in 0.1% TFA in water, run at a flow rate of 1 ml/min, and eluted with a linear gradient form 0-45% acetonitrile over 90 min. The labeled peptides were detected with an on-line model IC Flo-One Beta-radioactivity flow detector (Radiomatic Instruments, Inc., Tampa, FL), and collected as 1 ml fractions.

Phosphorylation Site Identification. Fractions corresponding to each labeled peptide were dried and further separated by electrophoresis on a 40% alkaline gel. Labeled peptides were detected by autoradiography of the dried gel, excised, and eluted with H_2O as previously described (11).

To find the position of phosphoamino acids in the peptides, I used manual Edman degradation as described by Sullivan and Wong (25). In brief, the peptide to be analyzed was dissolved in 30 ul of 50% acetonitrile, and spotted on an arylamine-Sequelon disc, which was placed on a Mylar sheet on top of a heating block set at 50°C. After 5 min, the aqueous solvent was evaporated and the disc was removed from the heating block. 5 ul of EDAC solution (50 mM in Mes, pH 5.0) was added to the disc to allow the peptide to covalently link to the disc, and the disc was placed at RT for 30 min. The disc was then washed five times with water and five times with TFA to remove unbound peptide. The disc was then washed three times with methanol, and subjected to Edman degradation: The disc was treated at 50°C for 10 min with 0.5 ml coupling reagent (methanol:water:triethylamine:phenylisothiocyanate; 7:1:1:1, v/v). After five washes with 1 ml of methanol, the disc was treated at 50°C for 6 min with 0.5 ml TFA to cleave the amino terminal amino acid. The TFA solution was placed in a scintillation vial and the disc was washed with 1 ml of TFA and 42.5% phosphoric acid (9:1, v/v). The wash was combined with the TFA solution and the released [^{32}P] was determined by Cerenkov counting. The next cycle began after the disc was washed five times with 1 ml methanol.

To characterize the peptides, the tryptic peptides were digested with the endoproteinases Glu-C and Asp-N. Glu-C cuts on the C-terminal side of Glu, except for Glu-Pro bonds. Moreover, Glu-X bonds within three residues of the end of a peptide are cleaved poorly (26). Asp-N cuts on the N-terminal side of Asp residues. Peptides, digested and undigested, were loaded to a peptide gel electrophoresis or manual Edman degradation. Glu-C digestion was performed in 200 ul 25 mM ammonium bicarbonate, pH 7.8, for 8 h at 37°C. Asp-N digestion was perfomed in 200 ul 50 mM sodium phosphate buffer, pH 8, containing 0.2 ug Asp-N and incubated at 37°C for 4 hours.

Site-directed Mutagenesis and Sequencing. Site-directed mutagenesis was perfomed using a kit from Clontech. Briefly, two primers were simultaneously annealed to one strand of the denatured double-stranded expression vector. The selection primer contains a mutation which changes a restriction site from Nde 1 to Afl II in the plasmid backbone. The mutagenesis primer changes Ser to either Ala, or Glu. After DNA elongation, ligation and a primary selection by digesting with restriction enzyme Nde 1, the mixture of mutated and unmutated plasmids were transformed into a mutS E. coli strain defective in mismatch repair. Plasmid DNA was enriched from the pool of transformants and and digested again with Nde 1 and transformed in DH5α bacterial strain. Transformants resistant to Nde 1 digestion were selected and plasmid DNA prepared and sequenced using a USB Sequenase Version 2.0 DNA Sequencing Kit based on chain-termination sequencing theory (27).

Transient transfection and CAT assays. Hela cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The day before transfection, 1 x10^6 cells were plated in 24 well plates and 4-6 h later switched to serum-free medium supplemented with Nutridoma-SR (Boehringer Mannhein). Cells were transfected with mammalian expression vectors encoding hPR or other factors as indicated in figure legends along with 1 ug of CAT reporter using the Lipofectamine method (GibcoBRL). 24 hr later, medium was replaced with fresh medium, and treated with hormone or other reagents as indicated in the figure. Cells were harvested and proteins prepared by three cycles of freeze-thaw lysis in the buffer (25 mM Tris-HCl, pH 8.0) 48 hr after transfection. The CAT activity was determined using a liquid CAT assay method (28).

Western Blot Analysis. Whole cell extracts of transfected cells are separated by SDS-gel Electrophoresis in a 10% acrylamide gel and electrotransferred to an nitrocellulars membrane. The membrane is probed with an hPR antibody recently generated, secondary rabbit antimouse IgG, and third peroxidase conjugated anti-rabbit IgG. The hPR bands are visualized by chemiluminesence using the ECL system (Pierce). The hPR is quantified by scanning laser densitometry.

B. Results

1. Characterization of hPR phosphotryptic peptides 10 and 11. Fig. 1 summarizes all the identified phosphorylation sites. The graph represents a reverse phase HPLC map of phosphotryptic peptides of hPR phosphorylated *in vivo* in the presence of hormone and a diagram of hPR structure. The location of phosphorylation sites and peptide peaks containing the sites are indicated by lines that connect both. Each phosphopeptide peak has been numbered based on the order of its retention time. To date, the only sites remaining to be identified are present in peptide 10(p11) and 11 (p11). In order to identify phosphorylation sites in each peak, HPLC fractions corresponding to each peak were collected and subjected to a 40% alkaline gel for further separation. Separated p10 and p11 were digested by specific proteinase Asp-N or Glu-C followed by 40% alkaline gel and autoradiographed. Asp-N and Glu-C cut, respectively, at the N-terminal side of Asp residues and the C-terminal side of Glu residues. p10 and p11 are cleaved by Glu-C (Fig. 2) but not by Asp-N (data not shown), suggesting that p10 and p11 contain cleavable Glu. Glu-C digestion of p10 and p11 resulted in two phosphopeptides, p10-1 and p10-2. The results suggest that p10 and p11 are related to each other because their patterns in gel before and after Glu-C treatment were the same. Manual Edman degradation experiments were performed to locate the phosphorylated residues. However after 30 cycles no ^{32}P was released. Since no peptide longer than 30 aa contains Ser (only Ser is phosphorylated in p10 and p11 according to our phosphoamino acids analysis), it is conceivable that the Edman degradation reaction is inhibited due to possibly cyclized glutamine at the amino terminal end of peptide.

In order to identify the phosphorylation sites, I used baculovirus expressed PR as a carrier to perform microsequencing. Baculovirus PR was digested with trypsin (5% trypsin to receptor, wt/wt). The tryptic peptides were separated by HPLC. The fraction corresponding to ^{32}P labeled phosphopeptide 10 and 11 was collected and subjected to automated sequencing. The sequencing data revealed the first 10 amino acids of a peptide beginning with Gln194:QLLLPASESPHWSGAPVK. There are three serines in the peptide. Since Glu-C digestion resulted in two phosphopeptides, I predict that at least two serines including Ser200 are phosphorylated. I plan to confirm the identification of Ser200 by treating the p10-1 with pyroglutamate aminopeptidase to remove cyclized Gln followed by manual Edman degradation. If Ser200 is the phosphorylation site, ^{32}P will be released at cycle 6. Other site(s) in p10-2 can be identified through performing manual Edman degradation directly on p10-2.

2. The effect of some kinase and phosphatase modulators on the RU 486 antagonist to agonist switch. It has been shown that treatment with 8-Br cAMP and RU 486 together results in an agonist effect. It was not known, however, whether other signalings can cause the same effect. Thus, we proposed to test growth factor EGF, phosphotyrosine phosphatase inhibitor vanadate, and h-ras oncogene since they are involved in variety of signal transduction pathways. In addition, we were also interested in testing if the switch is specific for hPR-B or hPR-A. Since the original paper used a breast cancer cell line B-11 containing integrated MMTVCAT, I started my experiments

with B-11. The results (Fig. 3) were similar to what had been reported. However, this clone was not stable and after a few weeks, the activity was lost. Therefore, I decided to transiently transfect reporter gene along with receptor vector to Hela cells, which has no endogenous PR, to approach the problem. Fig. 4 and Fig. 5 demonstrate the effect of 8-Br cAMP on the switch using either hPR-B or hPR-A. The switch occurred when hPR-B was used but not hPR-A. Although in this system, RU 486 gave higher background, addition of 8-Br cAMP still increased the RU 486 agonist activity significantly. This result was similar to what had been obtained from the B-11 cells. Using this system I have tested the effect of EGF (Fig. 6), vanadate (Fig. 7), TPA (Fig. 8), and h-ras oncogen (Fig. 9) on the switch. Interestingly, all agents tested potentiated the effect of hormone, but only EGF, and h-ras caused the switch. We are now in the process to make cell lines that contain stably transfected reporter. So that we can reduce the agonist activity of RU 486 by itself to better demonstrate the switch. In addition, we can use these cell lines to test phosphorylation mutants to see if phosphorylation of receptor is involved in the switch.

3. Site-directed Mutagenesis and Transcriptional Activity of Mutant PR. To elucidate the functional role of phosphorylation *in vivo*, we have mutated individually all the identified sites either to Ala (mimicking the unphosphorylate state of receptor) or Glu (mimicking the negatively charged receptor) to investigate the transcriptional activity of the mutants. Transcriptional activity of wild type and mutant were tested by transfecting the plasmids into HeLa with a MMTVCAT reporter, and subsequently determining the CAT activity. I previously reported that mutation of Ser81 to Ala significantly reduced the CAT activity within the linear range, suggesting that phosphorylation of this site is very important for the transcriptional activity of hPR. I next tested the mutant Ala400 (Ser400 mutated to Ala) and Glu400 (Ser400 mutated to Glu) because Ser400 is phosphorylated by Cdk2 in vitro and the site is near the AF1 domain. To best compare the activity of wild type and the mutants, I purified the plasmids of wild type and the mutants simultaneously, and two batches of wild type and mutants were evaluated. Protein level of wild type hPR and mutants were detected and quantitified by laser scanning densitometry. Fig. 10 shows that the activity of the Ala400 was significantly lower than that of the wild type while Glu400 behaves more like the wild type. The data are representative of three separate experiments. It is striking that the expression of Ala400 is very low compared to wild type and Glu400. Whether or not the difference was due to change in stability of the mutant will be examined in future experiments.

III Conclusion

Like other steroid receptors, hPR is highly phosphorylated in T47D breast cancer cells. Phosphorylation of the hPR involved multiple steps and many serine residues. The role of phosphorylation in modulating hPR functions and RU 486 antigonist/agonist switch has been investigated.

Based on our phosphotryptic mapping studies, we estimated that hPR may have at least nine phosphorylation sites. Previously, I reported the identification of eight phosphorylation sites. In this report, I have characterized the phosphopeptides p10 and p11, the last two peptides that had not been characterized. I conclude p10 and p11 were the same peptide and that Ser200 and likely another yet to be determined serine are phosphorylated. Additional experiments are required to identify the site.

Using a transient transfection system, I observed that in addition to 8-Br cAMP, EGF growth factor and ras oncogene were responsible for the agonist activity of RU 486 in hPR-B, suggesting that multiple signaling pathways can convert RU 486 to an agonist. Future experiments are to make stable cell lines with an integrated reporter to test other phosphorylation modulators.

To date, we have mutated all the phosphorylation sites and begun to test their transcriptional activity. It is rather a labor intensive task to fully characterize all these mutants because of the requirement of proper controls. I show in here that after the transcription activity of Ala400 mutant after normalization to protein level is significantly lower than that of the wild type. I found that the protein level of Ala is extremely lower than wild type despite same amount of cDNA was used in the experiments. It is possible that phosphorylation of Ser400 is important for the stability of hPR and we are in the process to test this possibility.

In summary, I have completed Specific Aim #1 with the exception that sites in p10 and p11 remain to be identified or verified. I have completed Specific Aim #2 by showing that there is no altered or new phosphorylation site in hPR caused by treatment with RU 486 and 8-Br cAMP. I have also demonstrated that hPR-B is responsible for the switch. Finally, I have tested the effect of several phosphorylation modulators on the switch and demonstrate in this report that more than one pathway could cause the switch. To date, the role of phosphorylation in hPR function still remains largely unknown. Despite the fact that multiple sites have been identified, only a few hPR phosphorylation mutants have been characterized. Our data thus far suggest that phosphorylation is important for hPR activity.

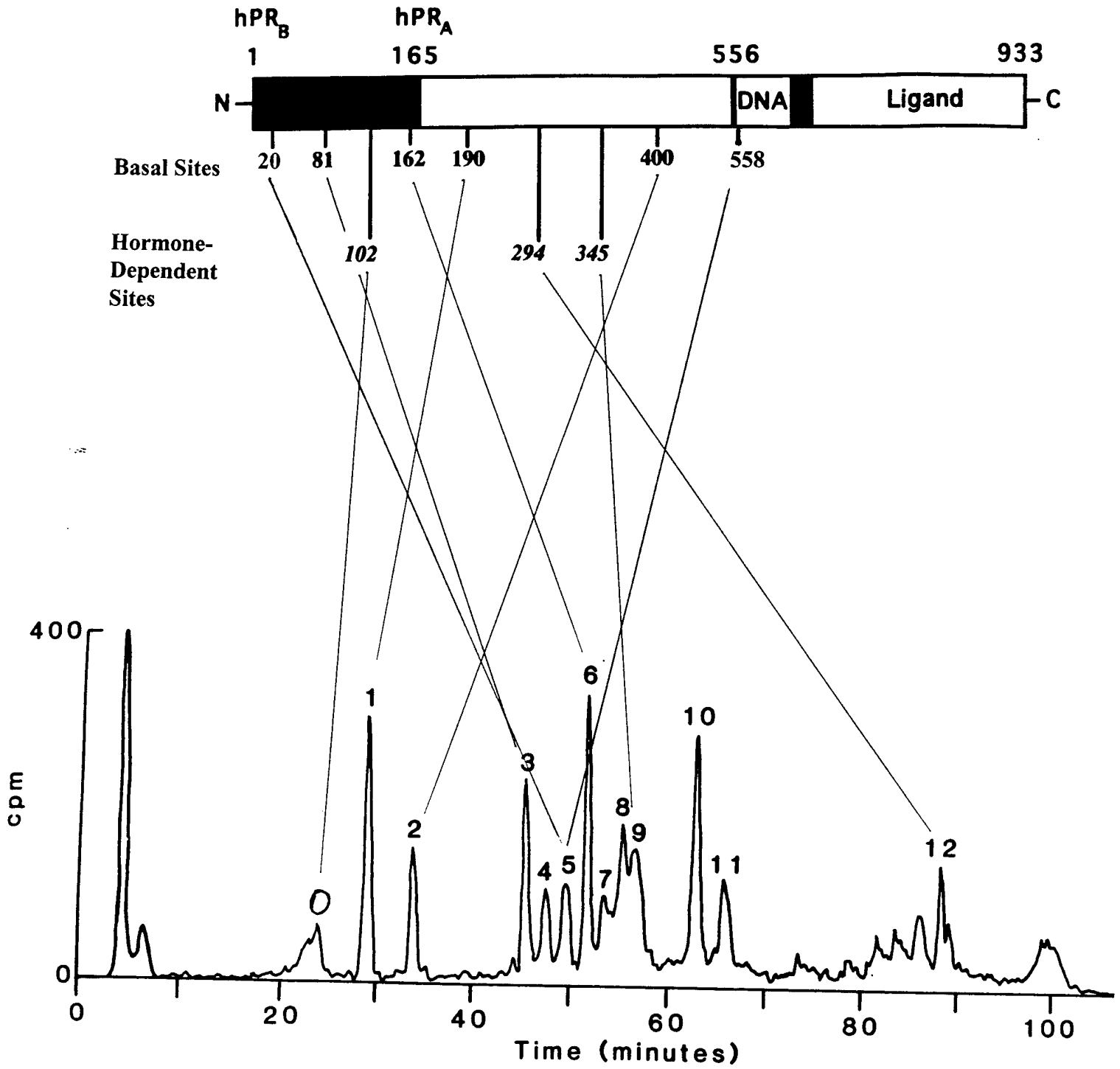


Figure 1. Summary of Human PR Phosphorylation Sites. The top panel shows a diagram of human PR. The lower panel represents the reverse phase HPLC profile of the tryptic phosphopeptides. Phosphorylation sites and their corresponding peaks are indicated by lines connecting the two. Two of the hormone-inducible sites (Ser294 and Ser345) are common to the A and B forms of PR, while Ser102 is unique to hPR-B. Ser20, Ser81 and Ser162 are previously identified sites that reside in the unique N-terminal segment of hPR-B and both are characterized as basal sites whereas Ser190, Ser400 and Ser558 are basal sites common to both hPR-A and hPR-B.

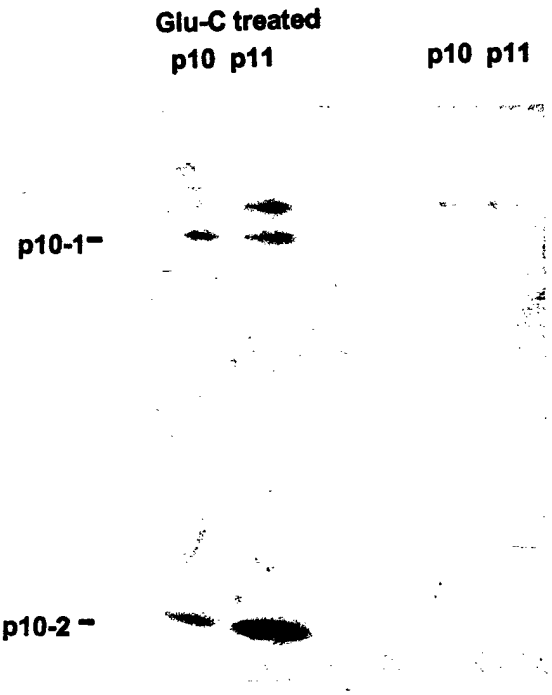


Figure 2. Characterization of phosphotryptic peptides p10 and p11. HPLC fractions corresponding to peak 10 (p11) and 11 (p11) were collected, a portion of both digested with Glu-C, all samples dried in a speedvac. Dried samples including digested and undigested were separated by a 40% alkaline gel and peptides were detected by autoradiography.

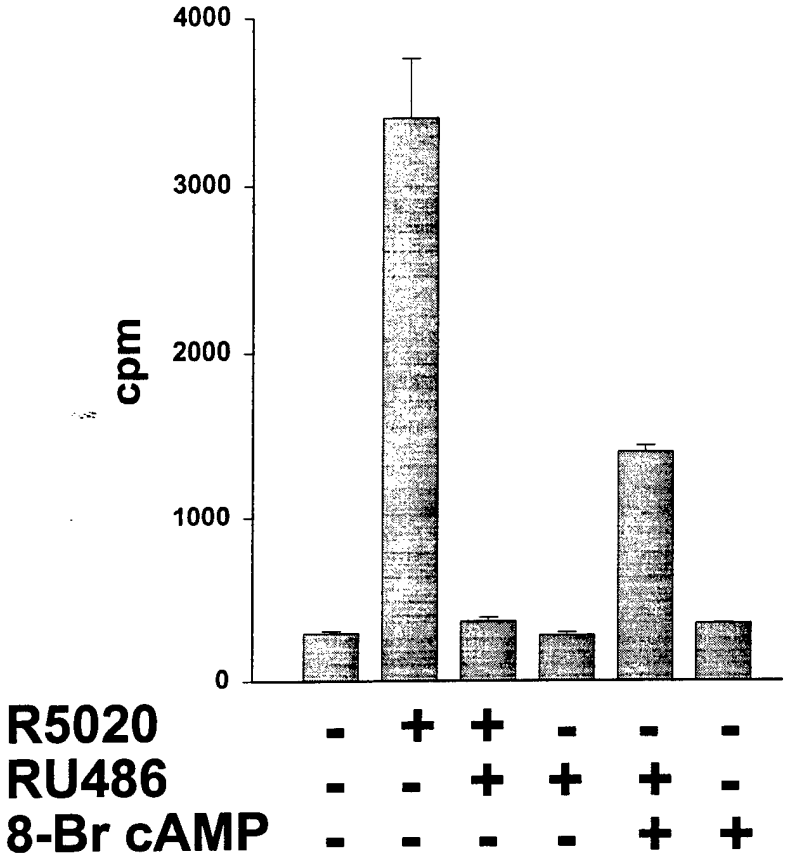


Figure 3. Transcriptional activity of hPR in the presence of RU 486 and 8-Br cAMP in breast cancer B-11 cells. Cells (2 x 10^5 cells/well) were treated as marked with 10 nM R5020, 10 nM RU 486, or 1 mM 8-Br cAMP. After 24 hrs, cells were harvested and protein levels were determined. Equal amount of protein was used to determine CAT activity using a liquid assay method. Samples were done in triplicate, and data are presented $\pm$ SEM.

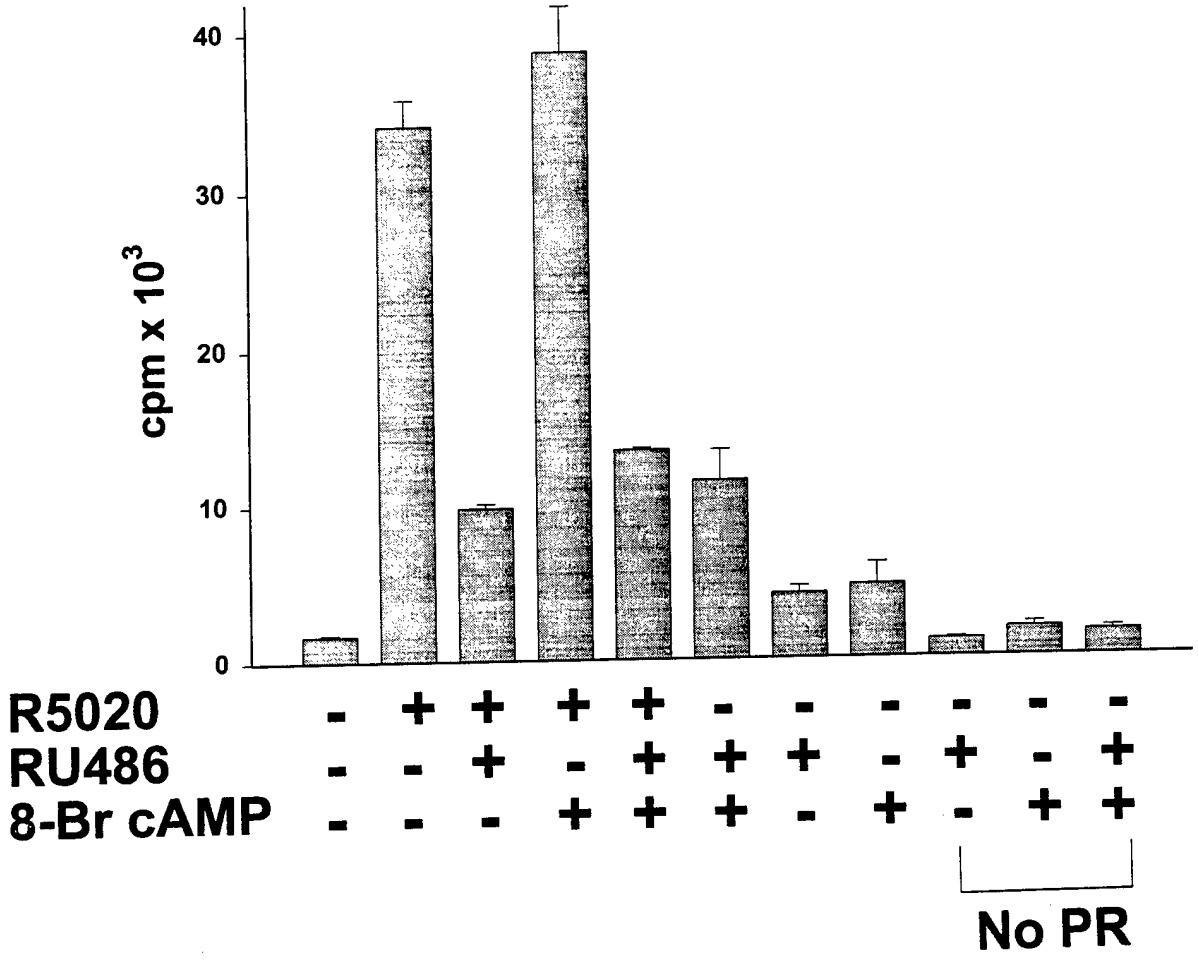


Figure 4. Transcriptional activity of hPR-B in the presence of RU 486 and 8-Br cAMP in HeLa cells. Cells (2 x 10^5 cells/well) were transfected with or without 0.1 ug hPR-B expression vector and 0.5 ug MMTVCAT reporter as designated using lipofectamine method. After 24 hrs, cells were treated as marked with 10 nM R5020, 10 nM RU 486, or 1 mM 8-Br cAMP. Cells were harvested 24 hrs later, equal amount of protein was used to determined CAT activity using a liquid assay. Samples were done in triplicate, and data are presented $\pm$ SEM.

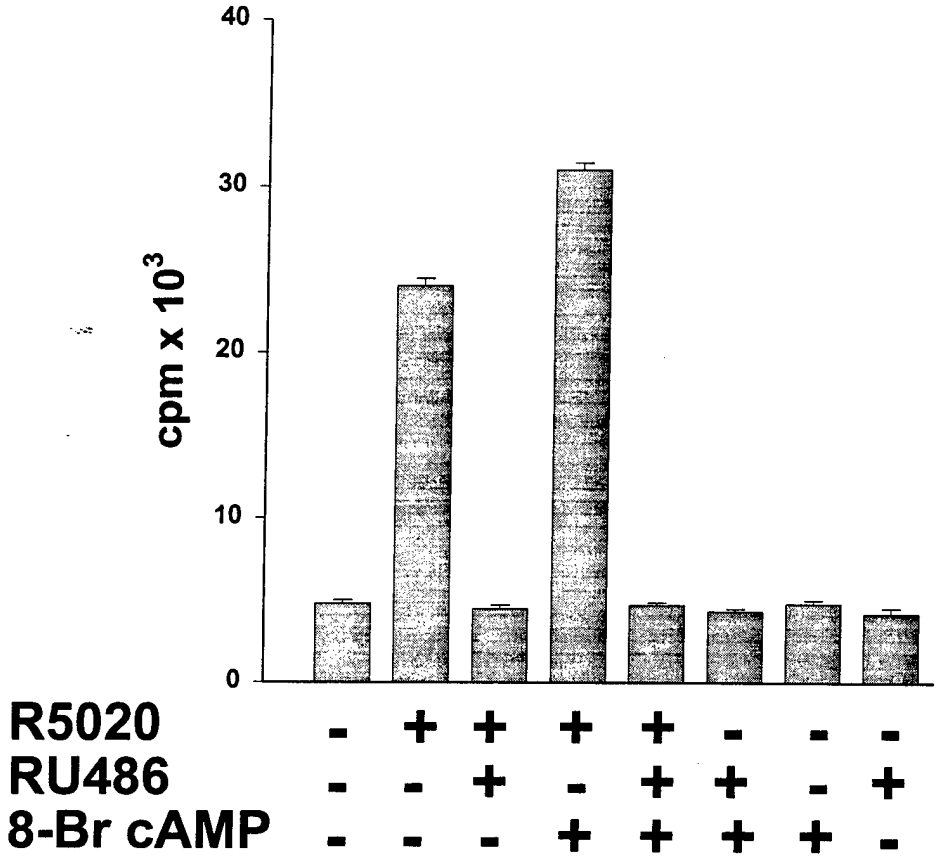


Figure 5. Transcriptional activity of hPR-A in the presence of RU 486 and 8-Br cAMP in HeLa cells. Cells (2 x 10^5 cells/well) were transfected with 0.25 ug hPR-A expression vector and 0.5 ug MMTVCAT reporter using lipofectamine method. After 24 hrs, cells were treated as marked with 10 nM R5020, 10 nM RU 486, or 1 mM 8-Br cAMP. Cells were harvested 24 hrs later, equal amount of protein was used to determined CAT activity using a liquid assay method. Samples were done in triplicate, and data are presented $\pm$ SEM.

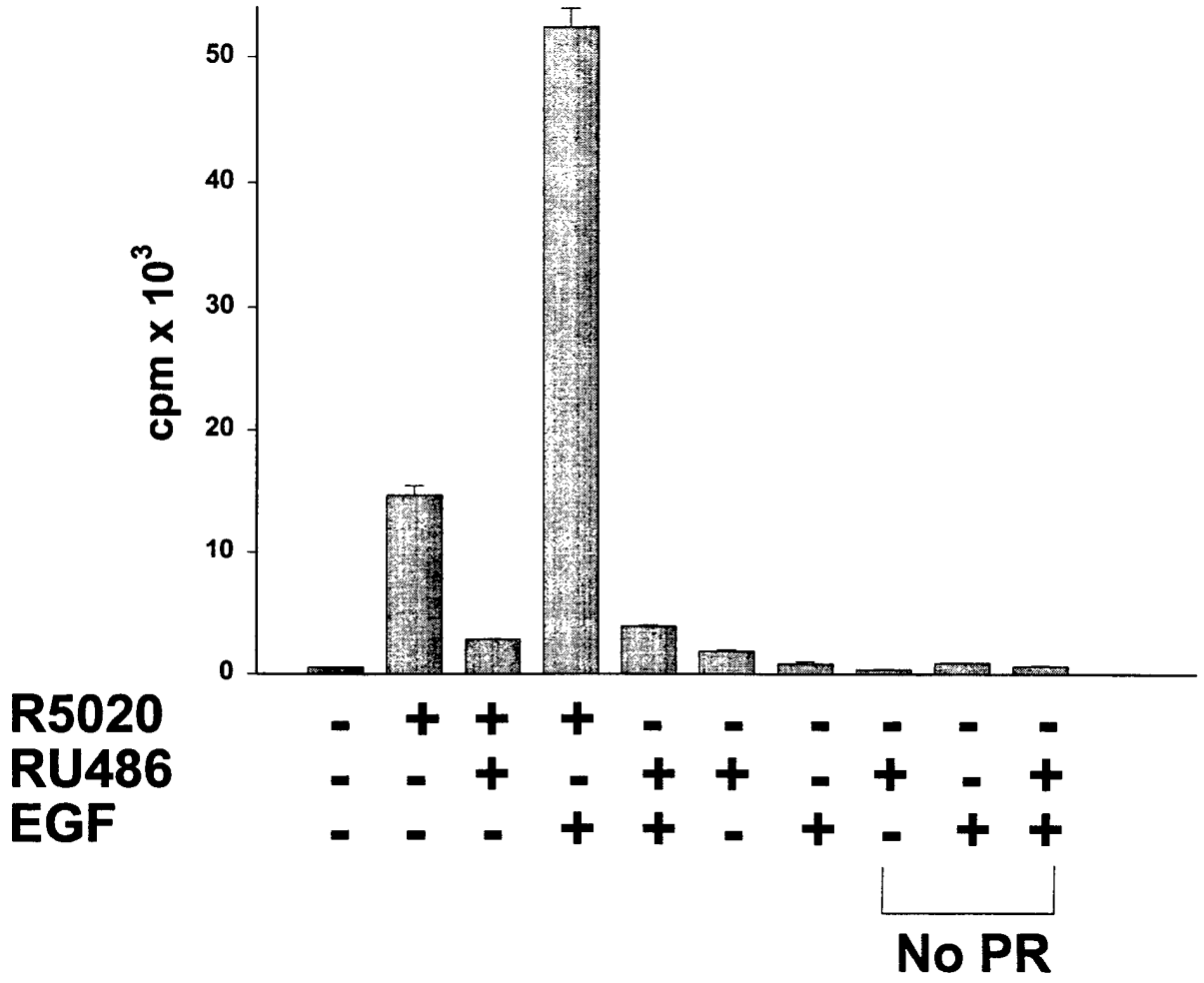


Figure 6. Transcriptional activity of hPR-B in the presence of RU 486 and EGF in HeLa cells. Cells (2 x 10^5 cells/well) were transfected with or without 0.1 ug hPR-B expression vector and 0.5 ug MMTVCAT reporter as designated using lipofectamine method. After 24 hrs, cells were treated as marked with 10 nM R5020, 10 nM RU 486, or 5 ng/ml human EGF. Cells were harvested 24 hrs later, equal amount of protein was used to determined CAT activity using a liquid assay method. Samples were done in triplicate, and data are presented $\pm$ SEM.

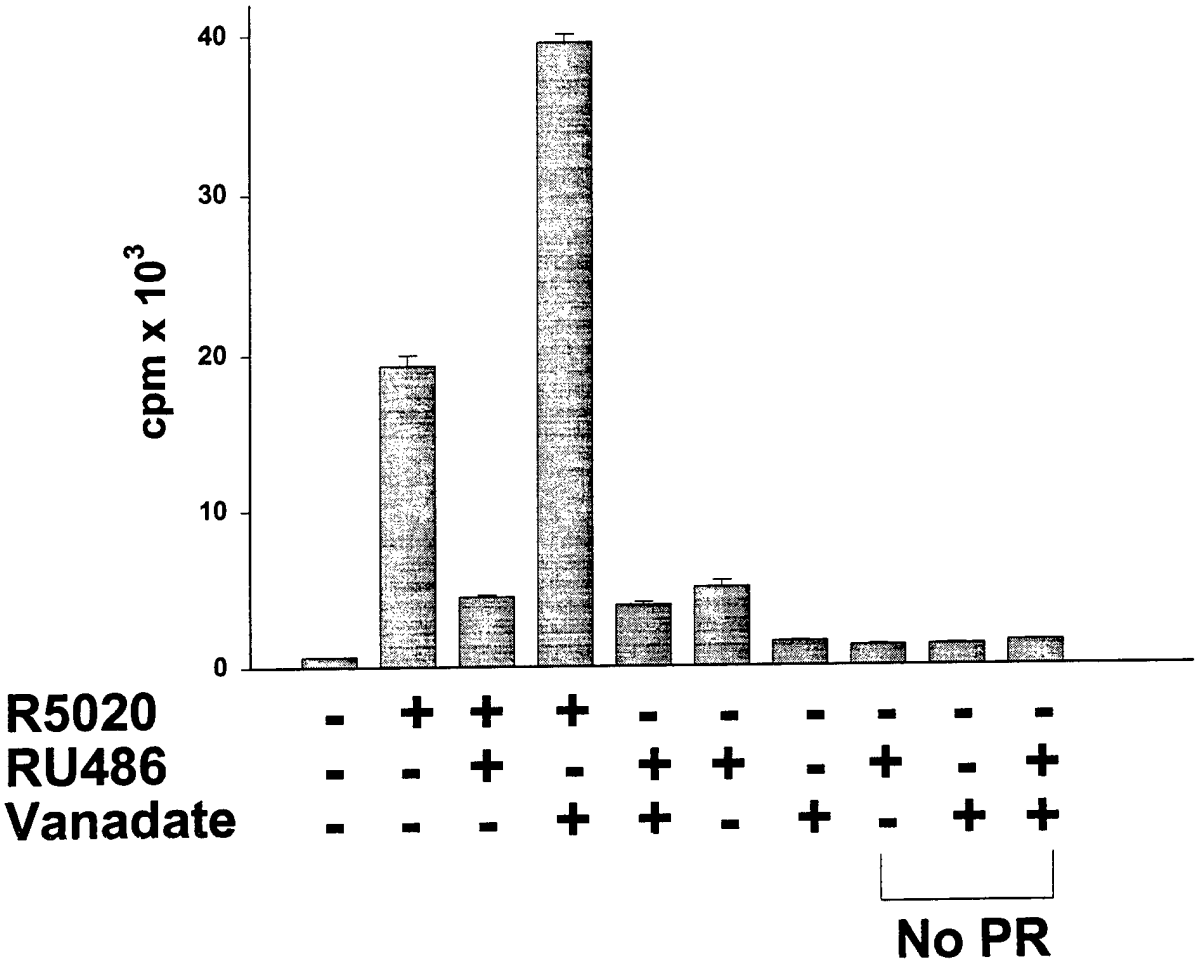


Figure 7. Transcriptional activity of hPR-B in the presence of RU 486 and vanadate in HeLa cells. Cells (2 x 10^5 cells/well) were transfected with or without 0.1 ug hPR-B expression vector and 0.5 ug MMTVCAT reporter as designated using lipofectamine method. After 24 hrs, cells were treated as marked with 10 nM R5020, 10 nM RU 486, or 25 uM vanadate. Cells were harvested 24 hrs later, equal amount of protein was used to determined CAT activity using a liquid assay method. Samples were done in triplicate, and data are presented $\pm$ SEM.

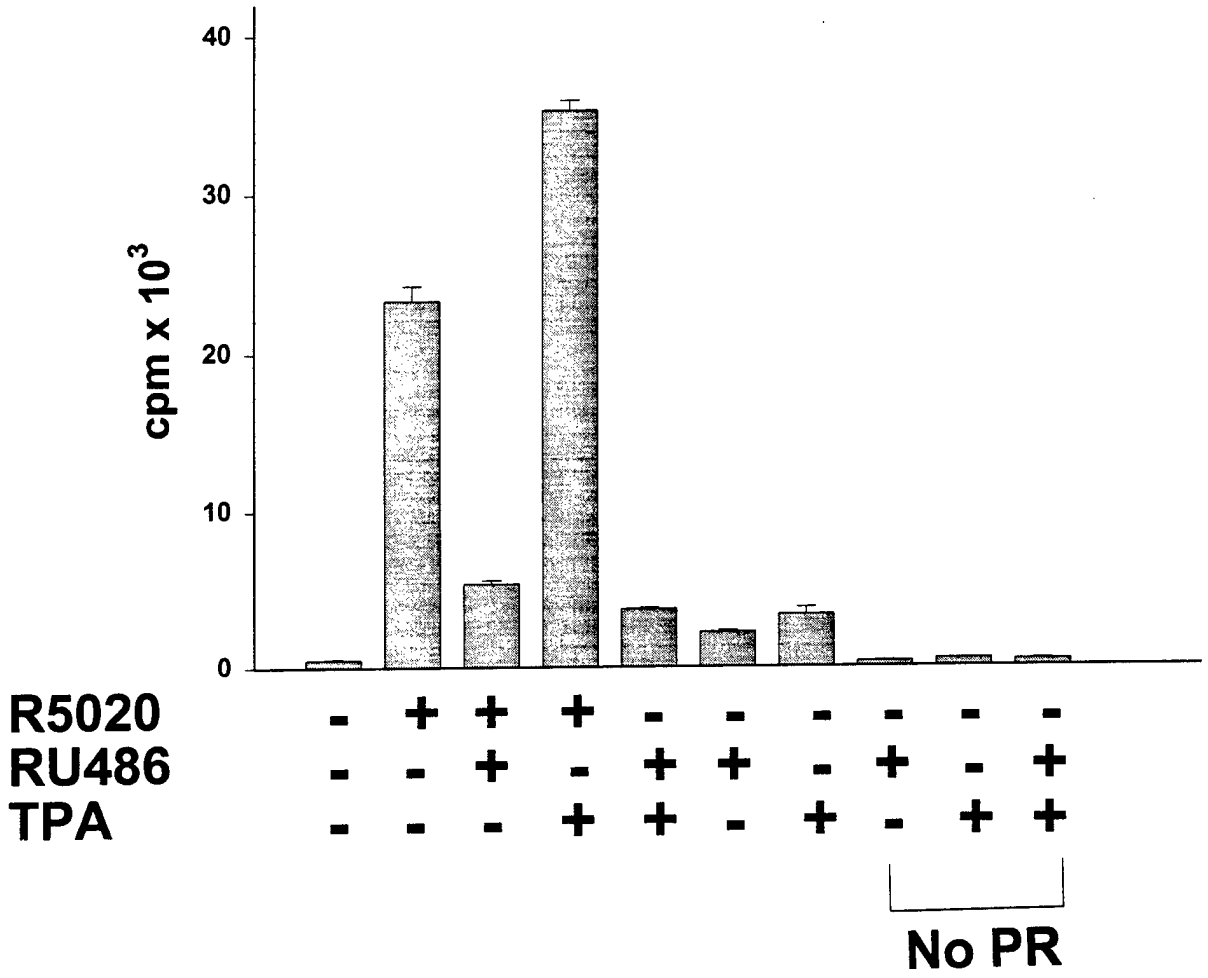


Figure 8. Transcriptional activity of hPR-B in the presence of RU 486 and TPA in HeLa cells. Cells (2 x 10^5 cells/well) were transfected with or without 0.1 ug hPR-B expression vector and 0.5 ug MMTVCAT reporter as desinated using lipofectamine method. After 24 hrs, cells were treated as marked with 10 nM R5020, 10 nM RU 486, or 50 nM TPA. Cells were harvested 24 hrs later, equal amount of protein was used to determined CAT activity using a liquid assay method. Samples were done in triplicate, and data are presented $\pm$ SEM.

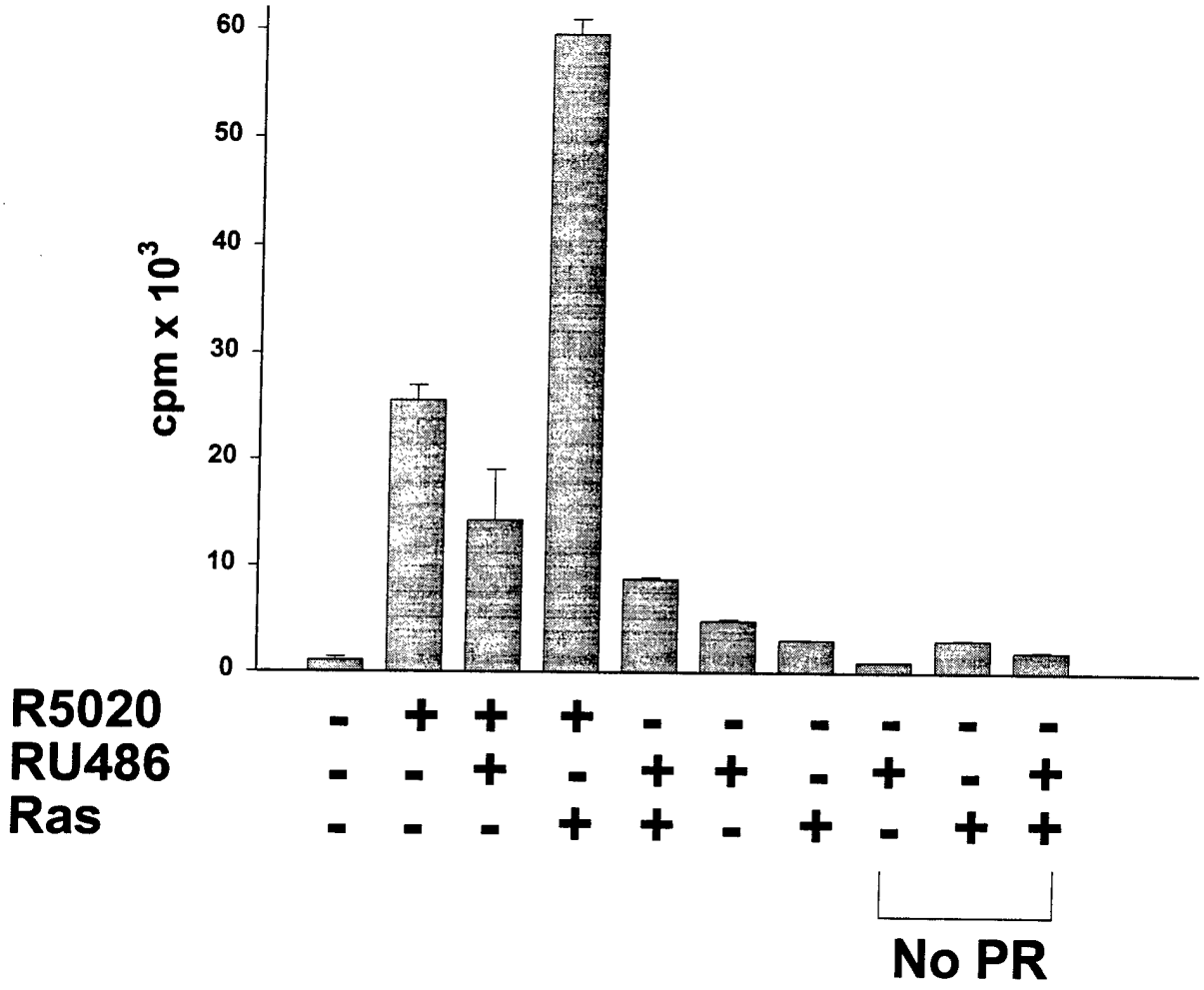
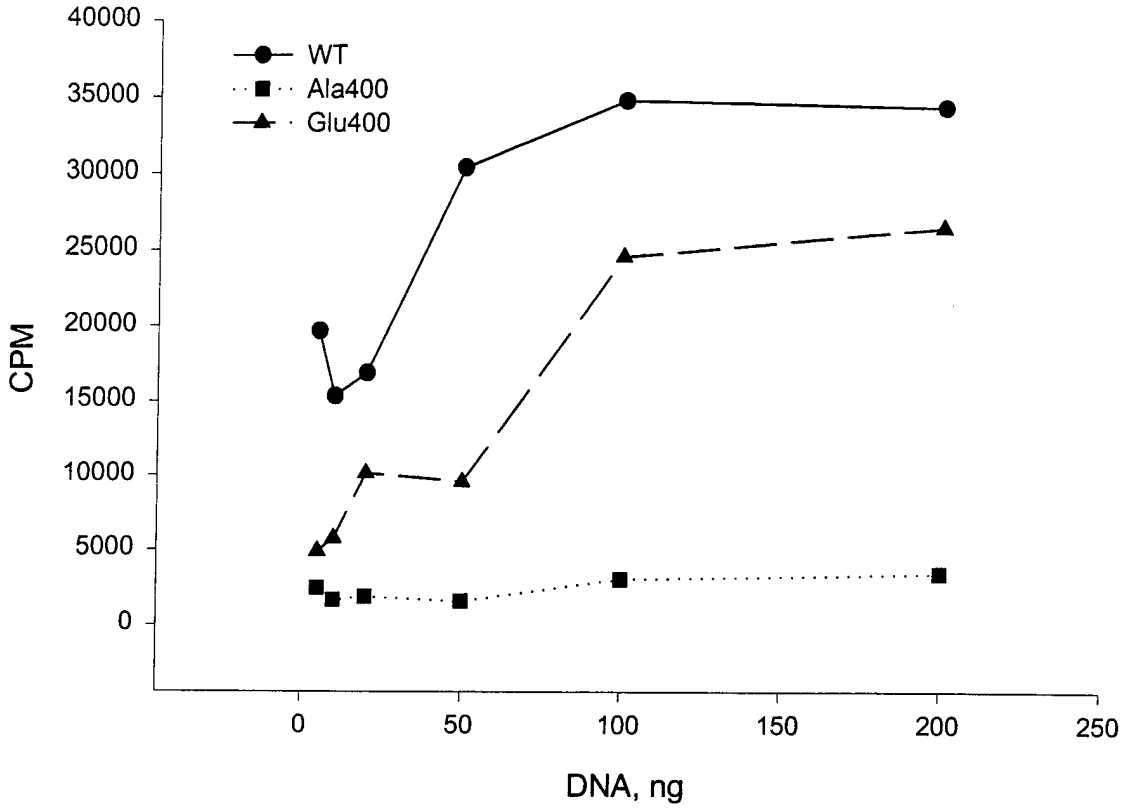


Figure 9. Transcriptional activity of hPR-B in the presence of RU 486 and h-ras in HeLa cells. Cells (2 x 10^5 cells/well) were transfected with 0.1 ug hPR-B expression vector, 0.1 h-ras expression vector, or 0.5 ug MMTVCAT reporter as desinated using lipofectamine method. The cells that were not treated with h-ras received 0.1 ug of h-ras parental vector DNA to make sure that vector itself does not contribute to the switch. After 24 hrs, cells were treated as indicated with 10 nM R5020, 10 nM RU 486. Cells were harvested 24 hrs later, equal amount of protein was used to determined CAT activity using a liquid assay. Samples were done in triplicate, and data are presented $\pm$ SEM.

A



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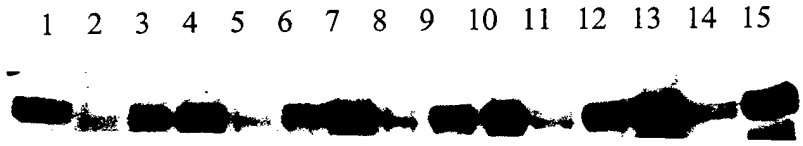

C

DNA, ng	CPM	Scan	Corrected	Avg.	SEM
WT					
5	17182				
10	19648	7469	2.63	2.2	0.07
20	20358	10074	2.02		
50	31880	14508	2.2		
100	35916	15026	2.39		
200	35586	19939	1.78		
Ala400					
5	2918				
10	3488	4397	0.79	0.8	0.02
20	2974	4494	0.66		
50	4034	5449	0.74		
100	4744	5466	0.87		
200	5774	6127	0.94		
Glu400					
5	8296				
10	8880	6722	1.32	1.7	0.1
20	11984	7277	1.65		
50	13278	10724	1.24		
100	28954	12593	2.3		
200	32040	14974	2.14		

Figure 10. Mutation of the hPR-B phosphorylation site, Ser400 to Ala decreased transcriptional activity of the receptor. Cells (2 x 10^5 cells/well) were transfected with lipofectamine bearing the indicated amount of receptor DNA and 0.5 ug MMTVCAT reporter. After 24 hrs, R5020 (10^{-8} M) was added, cells were harvested 24 hrs later and CAT activity determined by a liquid assay procedure. The expression of wild type and mutants were detected by western and their levels were quantitified with a scan densitometry. **Panel A:** CAT activity of wild type, Ala400, and Glu400. **Panel B:** western analysis of wild type, Ala400, and Glu400. **Panel C:** CAT activity is normalized to corresponding protein level and data are presented $\pm$ SEM.

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